Review

Genetic analyses of adaptin function from yeast to mammals

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Abstract

Adaptor protein (AP) complexes are heterotetrameric assemblies of subunits named adaptins. Four AP complexes, termed AP-1, AP-2, AP-3, and AP-4, have been described in various eukaryotic organisms. Biochemical and morphological evidence indicates that AP complexes play roles in the formation of vesicular transport intermediates and the selection of cargo molecules for inclusion into these intermediates. This understanding is being expanded by the application of genetic interference procedures. Here, we review recent progress in the genetic analysis of the function of AP complexes, focusing on studies that make use of targeted interference or naturally-occurring mutations in various model organisms. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

The exchange of materials among the *trans*-Golgi network (TGN), endosomes, lysosomes and the plasma membrane is largely mediated by vesicles covered with a dense protein coat. The coat is thought to facilitate vesicle budding and to select cargo molecules for incorporation into the vesicles. Coats containing the protein clathrin are the best characterized. They consist of an outer shell of clathrin and an inner shell containing either one of two heterotetrameric adaptor protein (AP) complexes, AP-1 or AP-2 (reviewed by Hirst and Robinson, 1998; Le Borgne and Hoflack, 1998a; Kirchhausen, 2000; Brodsky et al., 2001). The AP complexes mediate both the recruitment of clathrin to membranes and the recognition of sorting signals within the cytosolic tails of transmembrane cargo molecules. Several types of sorting signals have been described, the most common of which are referred to as 'tyrosine-based' or 'di-leucine-based' depending on the residues critical for activity (reviewed by Bonifacino and Dell'Angelica, 1999; Kirchhausen, 1999).

Two additional heterotetrameric AP complexes, AP-3 and AP-4, have been described recently (reviewed by Robinson and Bonifacino, 2001). Mammalian (but not yeast) AP-3 has been shown to interact with clathrin (Dell'Angelica et al., 1998; Drake et al., 2000; Liu et al., 2001), although it is not enriched in purified clathrin-coated vesicles (Simpson et al., 1997) and is able to promote synaptic vesicle budding in vitro in a clathrin-independent fashion (Faundez et al., 1998). AP-4 is most likely part of a non-clathrin coat (Dell'Angelica et al., 1999a; Hirst et al., 1999). The nature of the outer shell of AP-3- and AP-4-containing coats thus remains to be elucidated.

AP complexes are widely distributed among eukaryotes. The four basic AP complexes exist in Homo sapiens and Mus musculus, as well as in the plant *Arabidopsis thaliana*. The yeast *Saccharomyces cerevisiae*, the nematode *Caenorhabditis elegans*, and the fruit fly *Drosophila melanogaster*, on the other hand, possess only three AP complexes homologous to the mammalian AP-1, AP-2, and AP-3 (reviewed by Boehm and Bonifacino, 2001).

Each AP complex is composed of two large chains (one each of $\gamma/\alpha/\delta/\epsilon$ and $\beta1$–4, respectively, 90–130 kD), one medium-sized chain ($\mu1$–4, ~50 kD), and one small chain ($\sigma1$–4, ~20 kD) (Fig. 1, Table 1, reviewed by Lewin and Mellman, 1998; Kirchhausen, 1999; Robinson and Bonifacino, 2001). The subunits of AP complexes are collectively referred to as 'adaptins'. Many of the adaptins occur in two or more closely related isoforms encoded by distinct genes.
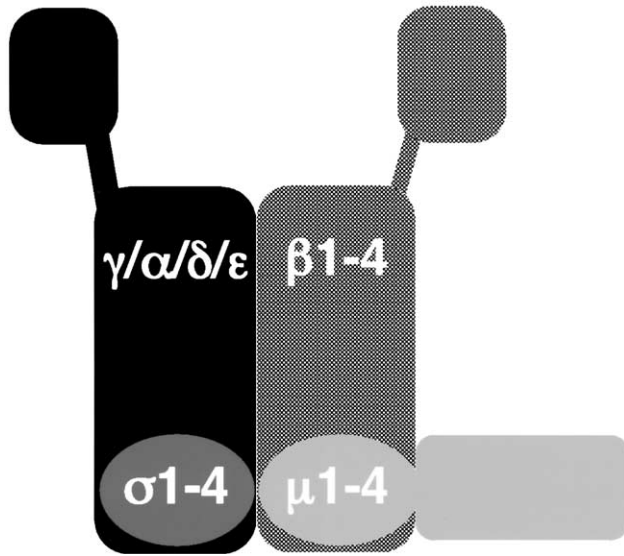
Abbreviations: ALP, alkaline phosphatase; AP complex, adaptor protein complex; CPS, carboxypeptidase S; CPY, carboxypeptidase Y; ER, endoplasmic reticulum; GFP, green fluorescent protein; GGAs, Golgi-localizing, gamma-adaptin ear homology, ARF-binding proteins; HPS, Hermansky-Pudlak syndrome; LDL, low density lipoprotein; mRNA, messenger RNA; pc, post-coitum; RNAi, RNA-mediated interference; TGN, trans-Golgi network

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Fig. 1. Schematic representation of a prototypic AP complex with two large subunits (γ/α/δ/ε and β1–4, 90–130 kD, respectively), one medium-sized subunit (μ1–4, ~50 kD), and one small subunit (σ1–4, ~20 kD).

Most of the mammalian isoforms, such as γ1, γ2, σ1A, σ1B, α1, α2, σ3A, and σ3B are expressed ubiquitously. The exceptions are β3B and μ3B, which are expressed only in brain (Pevsner et al., 1994; Newman et al., 1995),

and μ1B, which is specifically expressed in polarized epithelial cells (Ohno et al., 1999).

Many valuable insights into the function of AP complexes have been gleaned from the study of their subcellular localization and interactions with other proteins. AP-2 appears to be almost exclusively localized to plasma membrane clathrin-coated pits and derived clathrin-coated vesicles. This complex interacts with internalization signals present in the cytosolic tails of endocytic receptors, consistent with a role in rapid internalization. AP-1, AP-3, and AP-4, on the other hand, localize to intracellular compartments such as the TGN and/or endosomes. These complexes also interact with signals in the cytosolic tails of transmembrane proteins, but the exact correspondence between these interactions and specific sorting events remains to be determined.

In recent years, the study of the physiological roles of AP complexes has been greatly aided by the application of genetic manipulation methodologies. The conservation of the protein trafficking machinery as well as the high degree of homology of AP complexes from yeast to mammals has allowed general insights to be obtained from genetic analyses in organisms such as *S. cerevisiae*, *C. elegans*, *D. melanogaster*, *M. musculus*, and *H. sapiens*. In this article, we review recent progress in the understanding of AP complex function at the cellular and organismal levels derived from these genetic analyses.

Table 1
AP complex genes (and proteins) identified in various eukaryotes

Complex	Subunit	*H. sapiens*	*M. musculus*	*D. melanogaster*	*C. elegans*	*S. cerevisiae*
AP-1	Large	*ap1g1* (γ1)	*ap1g1* (γ1)	*cg9113* (γ)	*y105e8e.j* (γ)	*APL4* (Apl4p)
		ap1g2 (γ2)	*ap1g2* (γ2)			
		ap1b1 (β1)	*ap1b1* (β1)	*cg12532* (β1/2[a])	*y71h2b.10* (β1/2[a])	*APL2* (Apl2p)
	Medium	*ap1m1* (μ1A)	*ap1m1* (μ1A)	*cg9388* (μ1)	*k11d2.3* (Unc-101)	*APM1* (Apm1p)
		ap1m2 (μ1B)	*ap1m2* (μ1B)		*f55a12.7* (Apm-1)	*APM2* (Apm2p)
	Small	*ap1s1* (σ1A)	*ap1s1* (σ1A)	*cg5864* (σ1)	*f29g9.3* (σ1)	*APS1* (Aps1p)
		ap1s2 (σ1B)	*ap1s2* (σ1B)			
		ap1s3 (σ1C)	*ap1s3* (σ1C)			
AP-2	Large	*ap2a1* (α1)	*ap2a1* (α1)	*cg4260* (α)	*t20b5.1* (α)	*APL3* (Apl3p)
		ap2a2 (α2)	*ap2a2* (α2)			
		ap2b1 (β1)	*ap2b1* (β1)	*cg12532* (β1/2[a])	*y71h2b.10* (β1/2[a])	*APL1* (Apl1p)
	Medium	*ap2m1* (μ2)	*ap2m1* (μ2)	*cg7057* (μ2)	*r160.1* (Apm-2)	*APM4* (Apm4p)
	Small	*ap2s1* (σ2)	*ap2s1* (σ2)	*cg6056* (σ2)	*f02e8.3* (Aps-2)	*APS2* (Aps2p)
AP-3	Large	*ap3d1* (δ)	*ap3d* (δ)	*cg11197* (δ)	*w09g10.4* (δ)	*APL5* (Apl5p)
		ap3b1 (β3A)	*ap3b1* (β3A)	*cg11427* (β3)	*r11a5.1a* (β3)	*APL6* (Apl6p)
		ap3b2 (β3B)	*ap3b2* (β3B)			
	Medium	*ap3m1* (μ3A)	*ap3m1* (μ3A)	*cg3035* (μ3)	*f53h8.1* (μ3)	*APM3* (Apm3p)
		ap3m2 (μ3B)	*ap3m2* (μ3B)			
	Small	*ap3s1* (σ3A)	*ap3s1* (σ3A)	*cg3029* (σ3)	n/a (σ3)	*APS3* (Aps3p)
		ap3s2 (σ3B)	*ap3s2* (σ3B)			
AP-4	Large	*ap4e1* (ε)	*ap4e1* (ε)	n.p.[b]	n.p.[b]	n.p.[b]
		ap4b1 (β4)	*ap4b1* (β4)			
	Medium	*ap4m1* (μ4)	*ap4m1* (μ4)			
	Small	*ap4s1* (σ4)	*ap4s1* (σ4)			

[a] The β1/2 adaptins of *C. elegans* and *D. melanogaster* are most likely components of both AP-1 and AP-2.
[b] n.p.: The AP-4 complex is not present in these organisms.

2. AP-1

2.1. Genetic interactions of AP-1 with clathrin and the GGAs in S. cerevisiae

Because of the ease of genetic manipulation in the yeast *S. cerevisiae*, initial genetic analyses of AP complex function were carried out in this organism. Disruption of the genes encoding Aps1p, Apm1p, Apl2p, and Apl4p (the *S. cerevisiae* homologues of the σ1, μ1, β1, and γ subunits of AP-1, respectively) yielded no overt phenotype (Nakai et al., 1993; Phan et al., 1994; Rad et al., 1995; Stepp et al., 1995; Yeung et al., 1999). Haploid Aps1p, Apm1p, and Apl2p deletion strains (i.e. *aps1Δ*, *apm1Δ*, and *apl2Δ*) exhibited normal growth, sporulation, α-factor processing, and vacuolar protein sorting (Phan et al., 1994; Stepp et al., 1995). However, when tested in cells in which the heavy chain of clathrin was rendered temperature sensitive (*chc1^{ts}*), all AP-1 subunit disruptions displayed synthetic growth defects. While in the *chc1^{ts}* strain, growth at 37°C was only slowed, *chc1^{ts}/aps1Δ*, *chc1^{ts}/apm1Δ*, and *chc1^{ts}/apl2Δ* cells were unable to grow at this temperature (Rad et al., 1995).

In addition to a growth defect, a protein sorting defect also became apparent in cells lacking AP-1 subunit genes in combination with the *chc1^{ts}* allele (Phan et al., 1994; Rad et al., 1995; Stepp et al., 1995; Yeung et al., 1999). In *chc1^{ts}/apm1Δ* cells, for example, even at the growth permissive temperature of 25°C, more than 30% of the mating phero-mone α-factor was secreted as unprocessed pro-α-factor (Stepp et al., 1995). α-factor is synthesized as part of a larger precursor that is proteolytically cleaved by the endopeptidase Kex2p in the late Golgi complex (Fuller et al., 1988). Kex2p cycles between the late Golgi complex and a pre-vacuolar compartment. The impaired processing of pro-α-factor at 25°C in the *chc1^{ts}/apm1Δ* mutant over that seen in the *chc1^{ts}* cells suggests that Apm1p plays a role in the trafficking of Kex2p.

Another phenotypic characteristic of the *chc1^{ts}* mutant cells is a transient defect in sorting of carboxypeptidase Y (CPY) to the vacuole. In wild type cells, this soluble vacuolar protease is synthesized as a 67 kD p1 precursor form in the ER (Stevens et al., 1982). The p1 form is delivered to the Golgi complex where it undergoes addition of mannose residues to yield a 69 kD p2 form. The p2 form is subsequently sorted from the late Golgi complex to the vacuole by way of a pre-vacuolar compartment (Vida et al., 1993). After reaching the vacuole, the mature, active form is generated by proteolytic cleavage of the p2 form. In *chc1^{ts}* cells shifted to 37°C for short times, the p2 form was found to be transiently secreted into the culture medium instead of being sorted and proteo-lytically cleaved to mature CPY in the vacuole (Seeger and Payne, 1992). At longer times, however, the mutant cells adapted and resumed the proper sorting and processing of CPY. In *chc1^{ts}/aps1Δ/apl2Δ* cells, the initial CPY sorting defect was less accentuated than in the *chc1^{ts}* strain, thus indicating a genetic interaction of AP-1 with clathrin.

In line with this, both, Apl4p and Apl2p have been recently shown to mediate binding of AP-1 to clathrin, the latter through two clathrin-binding motifs in its carboxy-terminal region (Yeung et al., 1999; Yeung and Payne, 2001). When the genes encoding the endogenous proteins were replaced by mutated genes that rendered Apl2p and Apl4p incapable of binding clathrin, the growth and α-factor maturation defects in *chc1^{ts}* cells were accentuated, although not to the extent observed when AP-1 was deleted altogether.

Thus, in *S. cerevisiae* AP-1 appears to function together with clathrin to mediate protein sorting in the late Golgi complex. This function, however, is not essential for viability and the effects of mutating AP-1 are much less severe than those of mutating clathrin. Since clathrin is unable to bind to membranes by itself, this hints at the existence of additional clathrin adaptors in *S. cerevisiae*. Recent studies indicate that the GGA proteins Gga1p and Gga2p may serve such a role (Black and Pelham, 2000; Dell'Angelica et al., 2000b; Hirst et al., 2000, 2001; Costaguta et al., 2001; Mullins and Bonifacino, 2001; Zhdankina et al., 2001).

The GGA proteins, like AP-1, bind clathrin via sequence motifs in their carboxy-terminal domains. Double *gga* mutants (Costaguta et al., 2001; Dell'Angelica et al., 2000b) and *apl4Δ/gga1Δ/gga2Δ* cells grew at normal rates (Hirst et al., 2001) and the CPY sorting defect observed in the double *gga* deletion strain was not exacer-bated in cells that also lacked the Apl4p protein (Hirst et al., 2001). Synthetic interactions of the GGA proteins, however, with both clathrin and AP-1 subunits were detected in combined knock-outs of Apl2p and Gga2p as well as in triple mutants carrying mutations of both GGA proteins and either clathrin or the Apl2p subunit of AP-1. While the former showed a strong α-factor and pro-CPY matura-tion defect, the latter displayed reduced growth (Costaguta et al., 2001). No sorting defect for alkaline phosphatase (ALP, see Section 4.1), however, was apparent in these cells (Costaguta et al., 2001).

These observations suggest that AP-1 and the GGAs act in concert with clathrin to mediate protein sorting at the late Golgi complex. Some functions required for cell viability and/or normal growth appear to be redundant. The identifi-cation of cargo molecules that interact specifically with either AP-1 or the GGA proteins in *S. cerevisiae* may provide further clues to the nature of the distinct and over-lapping functions of these proteins.

2.2. Developmental defects in C. elegans mutants deficient in AP-1

The lack of a detectable phenotype in yeast mutants defi-cient in AP-1 alone would suggest that this complex is rela-tively unimportant for cell physiology. Studies in higher eukaryotes, however, indicate that AP-1 is essential for the development of multicellular organisms. *C. elegans* expresses two μ1 chain homologues, Unc-101 and Apm-1, both of which are interchangeable components of the AP-1
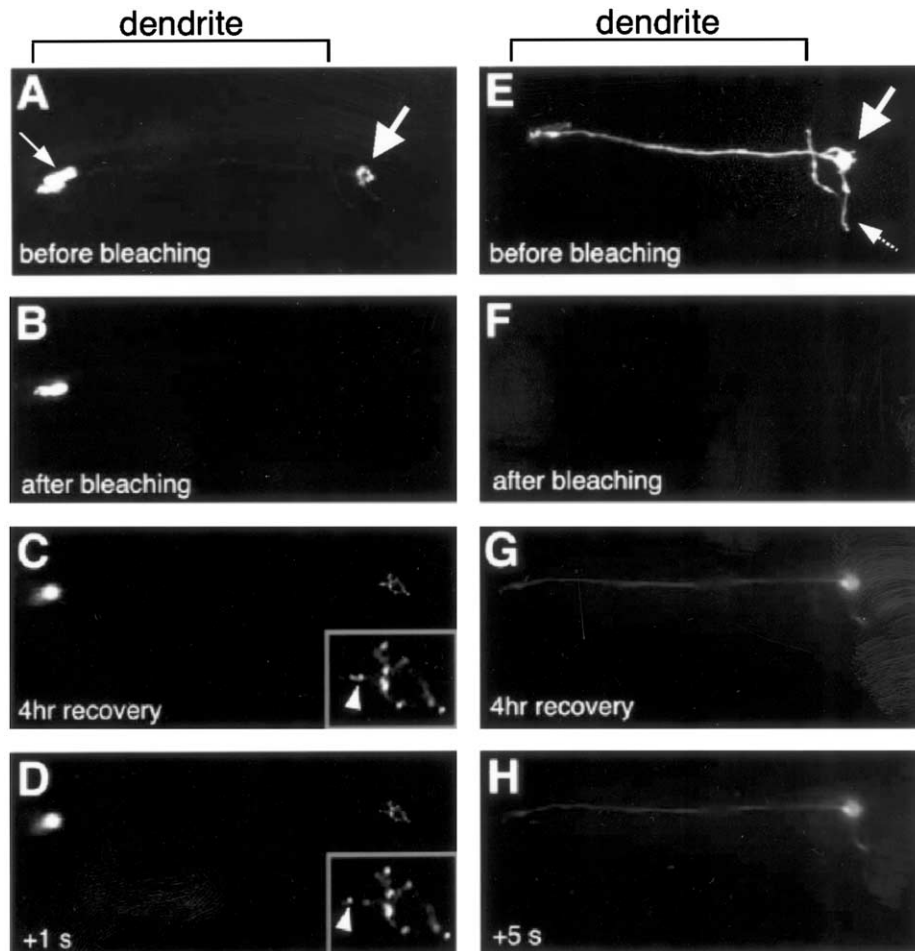
Fig. 2. Odr-10 is missorted and dendritic vesicles are not detected in *unc-101* mutants [modified from Neuron, Vol. 31, Dwyer, N.D. et al., Polarized Dendritic Transport and the AP-1 μ1 Clathrin Adaptor UNC-101 Localize Odorant Receptors to Olfactory Cilia, Pages 277–287, Copyright© (2001), with permission from Elsevier Science]. Dendritic Odr-10-GFP fluorescence was observed in wild type and *unc-101* animals following photobleaching of the ubiquitous plasma membrane fluorescence. No Odr-10 positive dendritic vesicles were observed in the *unc-101* animals, suggesting that AP-1 is required for the formation of dendritic vesicles at the TGN in *C. elegans* chemosensory neurons. The position of the cilia (thin arrow in panel A), dendrites, cell bodies (thick arrows in panels A and E), and axons (dashed arrow in panel E) are indicated. (A–D) Wild-type animal (A) before photobleaching, (B) after photobleaching, and (C, D) after recovery. (C) and (D) were taken 1 s apart. A vesicle is seen moving in an anterograde direction near the cell body (arrowheads, insets in panels C and D). (E–H) *unc-101* animal (E) before photobleaching, (F) after photobleaching, and (G, H) after recovery. (G) and (H) were taken 5 s apart. Although fluorescence recovered slowly, no moving vesicles were observed. Anterior is at left in all panels.

complex. The naturally-occurring *unc-101* mutation as well as double-stranded RNA interference (RNAi) approaches targeting Apm-1 and the other AP-1 subunits have been utilized to study the effects of AP-1 deficiency in *C. elegans*. Mutations in Unc-101 cause defects in neuronal dye uptake, uncoordinated movement, male spicule defect and a defecation defect that can be rescued by expression of the mouse μ1A protein. Null mutations are lethal in 50% of the animals (Lee et al., 1994). Recently, Unc-101 has been linked to the formation of dendritic vesicles at the TGN as well as the proper sorting of the odorant receptor Odr-10 to the sensory cilia of olfactory dendrites (Dwyer et al., 2001). Whereas in wild type animals the receptor is transported in dendritic vesicles from the cell body to the cilia, Unc-101-negative animals show even distribution of the receptor at the plasma membrane (Fig. 2E). Other cilia membrane proteins, such as the Osm-9 channel and the transmembrane guanylyl cyclase Odr-1, are also mislocalized in Unc-101 negative animals (Dwyer et al., 2001), suggesting that the Unc-101-containing AP-1 complex is implicated in protein sorting to the sensory cilia of chemosensory neurons in *C. elegans*.

Shim et al. (2000) used a double-stranded RNAi approach to assess the function of Apm-1 and the other subunits of AP-1 in *C. elegans*. Interference with Apm-1 resulted in all of the F1 progeny being arrested at the L1 larval stage with severe intestinal abnormalities. Interference with both μ1 subunits by RNAi proved to be embryonic lethal within the eggshell at 100%. This phenotype was also observed when RNAi was done on γ, β1 or σ1.

The results obtained in *C. elegans* thus demonstrate that AP-1 plays an essential role in the development of a multi-

cellular organism. They also emphasize the importance of AP subunit isoforms by demonstrating that Unc-101 and Apm-1 have only partially overlapping functions in development. Finally, they suggest a probable role for AP-1 in sorting proteins to transport vesicles destined for a specific domain of the plasma membrane.

2.3. Embryonic lethality in mice deficient in AP-1

Genetic analyses of AP-1 function in mice have provided further evidence for the importance of this complex for the development of multicellular organisms. Zizioli et al. (1999) disrupted the gene encoding γ1-adaptin in mice, but found no homozygous mutants among the progeny of heterozygous parents. Blastocysts of homozygous mutant mice developed normally until day 3.5 post-coitum (pc), but died at day 4.5 pc when the levels of maternally supplied γ1-adaptin decreased. Attempts to culture cells from day 3.5 pc blastocysts failed because the cells did not spread and proliferate.

Heterozygous mutant mice exhibited a 50% decrease in the amount of intracellular γ1-adaptin protein and were growth-retarded when compared to their wild type littermates. No free β1, μ1 or σ1 proteins or AP-1 subcomplexes lacking γ1 were found in the heterozygous mutant mice (Zizioli et al., 1999). Since the levels of all other AP-1 subunit mRNAs were normal, this suggested either translational repression or, more likely, decreased stability of unassembled AP-1 subunits.

Disruption of the gene encoding the μ1A subunit of AP-1 also yielded no homozygous mutant animals (Meyer et al., 2000). Unlike the γ1 mutant embryos, however, the μ1A mutant embryos survived until day 13.5 pc. This allowed the culture of fibroblasts from homozygous mutant embryos, which proved to be viable. Although no free γ1, β1 or σ1 subunits were present in the fibroblasts, a γ1-β1-σ1 subcomplex was detected that showed diffuse cytosolic staining. Furin and the two mannose 6-phosphate receptors involved in sorting lysosomal enzymes at the TGN had a more dispersed distribution in the cytoplasm of the μ1A-deficient cells (Meyer et al., 2000; Fölsch et al., 2001). This suggested a possible role for mammalian AP-1 in the retrieval of furin and the mannose 6-phosphate receptors from endosomes to the TGN.

The longer survival of μ1A-deficient embryos as compared to that of γ1-deficient embryos can most likely be attributed to the expression of μ1B, a closely related isoform of μ1A in cells of epithelial origin (Ohno et al., 1999). The assembly of an AP-1 complex in epithelial cells would allow for development to proceed through the first 13 days of embryogenesis until the absence of AP-1 in other cell lineages becomes fatal. This is supported by the fact that the expression of μ1B in μ1A-deficient cells restored the TGN membrane-binding capacity of AP-1 (Meyer et al., 2000). The γ2 isoform, although ubiquitously expressed, appears not to be capable of functionally substituting for γ1.

Studies taking advantage of the absence of μ1B in the polarized epithelial kidney cell line LLC-PK1 demonstrated that the epithelial cell-specific μ1B-containing AP-1B complex plays a critical role in protein targeting to the basolateral domain of the plasma membrane (Fölsch et al., 1999). When μ1B was expressed by transfection into LLC-PK1 cells, proper sorting of the transferrin and LDL receptors to the basolateral plasma membrane was restored (Fölsch et al., 1999). These experiments thus demonstrated a role for a variant of the AP-1 complex in transport to a specific plasma membrane domain of mammalian cells.

2.4. Summary: role of AP-1 in intracellular trafficking

The genetic analyses of AP-1 function all point to its role in protein sorting at the late-Golgi/TGN or endosomes, but it is still unclear what its precise function is. In *S. cerevisiae*, AP-1 seems to be involved in late-Golgi sorting events involving clathrin, with the prevacuolar compartment or vacuole as the likely destinations for the proteins sorted. This function, however, is highly redundant with that of the GGAs, and is dispensable for viability. In *C. elegans*, the available evidence suggests that AP-1 directs protein sorting to dendritic vesicles destined for a specific domain of the neuronal plasma membrane. In mammals, the AP-1A isoform has been implicated in the retrieval of mannose 6-phosphate receptors from the endosomal system to the TGN, and the AP-1B isoform in the sorting of integral membrane proteins from the TGN or endosomes to the basolateral plasma membrane domain of polarized epithelial cells. Unlike in *S. cerevisiae*, all of these functions appear to be essential for the development and viability of *C. elegans* and mice.

3. AP-2

3.1. Deletion of the genes encoding AP-2 subunits in S. cerevisiae results in no observable phenotype

S. cerevisiae AP-2 is more distantly related to mammalian AP-2 at the amino acid sequence level than *S. cerevisiae* AP-1 and AP-3 are to their mammalian counterparts (Cowles et al., 1997a; Panek et al., 1997; Boehm and Bonifacino, 2001). In addition, it is well established that mammalian AP-2 binds clathrin (reviewed by Kirchhausen, 1999, 2000), whereas *S. cerevisiae* AP-2 does not (Yeung et al., 1999). Disruption of the genes encoding *S. cerevisiae* AP-2 subunits had no effect on viability of the mutant strains (Huang et al., 1999; Yeung et al., 1999). Likewise, the disruptions had no effect on endocytosis of the α-factor receptor Ste2p (Phan et al., 1994; Rad et al., 1995; Huang et al., 1999), the turnover of the **a**-factor receptor Ste3p (Yeung et al., 1999), the processing of pro-α-factor, and protein sorting to the vacuole (Rad et al., 1995; Huang et al., 1999; Yeung et al., 1999). Finally, disruption of AP-2 genes did not exacerbate the defects observed in temperature-sensitive clathrin mutants (Rad et al., 1995). Thus, in

contrast to mammalian AP-2, *S. cerevisiae* AP-2 does not appear to interact either physically or functionally with clathrin. This has led Payne and colleagues to refer to this complex as AP-2R, with the 'R' meaning 'related' (Yeung et al., 1999). Therefore, *S. cerevisiae* AP-2 seems to be even less important for normal cell physiology than AP-1. The conservation of this complex in yeast, however, suggests that it must play some critical role under certain conditions.

3.2. Disruption of AP-2 subunit genes in C. elegans causes defects in endocytosis

Analogously to the differential requirement for AP-1 in yeast and in higher eukaryotes, AP-2 seems to be dispensable for viability in *S. cerevisiae* but required for normal cell function and embryonic development in higher eukaryotes. Grant and Hirsh (1999) examined the effect of RNA-mediated interference (RNAi) of AP-2 subunits on the uptake of a vitellogenin-GFP construct into oocytes of *C. elegans*. Vitellogenins are yolk proteins that are secreted as part of lipoprotein particles from the intestine into the body cavity, and subsequently taken up by clathrin-mediated endocytosis into the growing oocytes (Kimble and Sharrock, 1983; Hall et al., 1999). RNAi of α- or β-adaptin resulted in an inhibition of vitellogenin-GFP uptake (Grant and Hirsh, 1999). The oocytes of the RNAi-expressing animals were smaller relative to controls, and embryos were inviable. Surprisingly, RNAi of μ2 and σ2 had no effect on endocytosis and on the size of oocytes (Grant and Hirsh, 1999). μ2 and σ2 RNAi embryos did, however, show a severely *dumpy* phenotype – a short, fat body associated with defects in cuticle formation (Levy et al., 1993). This phenotype was possibly caused by a partial failure in embryonic elongation during morphogenesis (Grant and Hirsh, 1999). Another study by Shim and Lee (2000) showed that RNAi of μ2 and σ2 resulted in embryonic lethality, larval lethality and morphological defects. The reason for the different requirements for the α, β, μ2, and σ2 subunits of AP-2 for vitellogenin-GFP endocytosis is not apparent, since the four proteins are subunits of the same complex. It is possible that partial complexes could have different levels of activity, depending on what subunit is missing. Thus, AP-2 is essential for development in *C. elegans* probably due to the critical role of some of its subunits in endocytosis of plasma membrane receptors.

3.3. The lack of AP-2 in D. melanogaster results in paralyzed larvae devoid of synaptic vesicles

The rapid internalization of plasma membrane proteins is particularly critical for synaptic vesicle recycling. An involvement of AP-2 in this process was not manifest in RNAi studies of *C. elegans* probably because RNAi is not effective in neurons (Tavernarakis et al., 2000). Studies of α-adaptin mutants in *D. melanogaster*, however, did uncover a role for AP-2 in synaptic transmission. The role of α-adaptin in *D. melanogaster* was studied by P-element enhancer trap insertion (González-Gaitán and Jäckle, 1997). Disruption of the gene encoding α-adaptin resulted in homozygous embryos that developed into slowly moving larvae and died as pupae (D-αAda[1] allele). Imprecise excision of the P-element caused the appearance of two additional mutant alleles, D-αAda[2] and D-αAda[3]. D-αAda[2] mutants developed to adulthood, but the flies could neither walk nor fly. D-αAda[3] mutants were even more severely affected in that homozygous embryos died before hatching. All α-adaptin deficient embryos developed normally until the first instar larvae stage and the architecture of their nervous system was not affected. Muscle contractions, however, were only sporadic and the larvae did not hatch. At the cellular level, synapses of D-αAda[3] embryos were devoid of vesicles and plasma membrane coated pits (Fig. 3). Endocytosis was severely impaired, although not completely blocked, as judged by the delayed uptake of the lipophilic fluorescent dye FM1-43 into the boutons of presynaptic cells (González-Gaitán and Jäckle, 1997). All of these observations are consistent with a role for α-adaptin, and by extension of the AP-2 complex, in the recycling of synaptic vesicle membranes from the presynaptic surface.
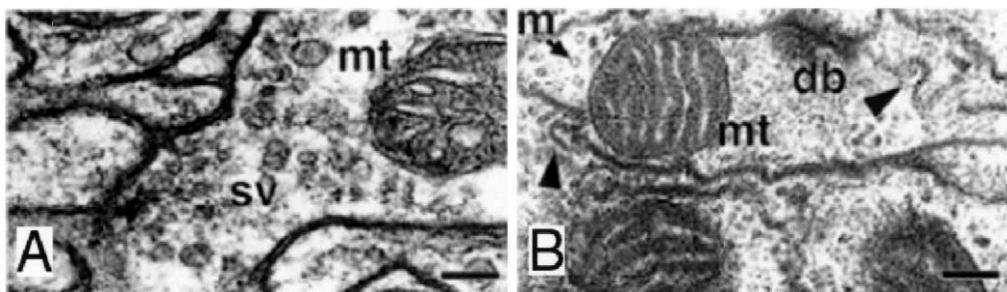

Fig. 3. D-αAdaptin mutations impair the formation of endocytotic synaptic vesicles [reprinted from Cell, Vol. 88, González-Gaitán, M. and Jäckle, H., Role of *Drosophila* α-Adaptin in Presynaptic Vesicle Recycling, Pages 767–776, Copyright© (1997), with permission from Elsevier Science]. Ultrastructure of a presynaptic terminal in the CNS neuropile of (A) wild type; and (B) homozygous D-αAda[3] mutant embryos. Note vesicles in the synaptic boutons of wild-type embryos that are absent in the mutant and also the deep folds of the presynaptic membrane of the mutant [arrowheads in (B)]. db, dense body; mt, mitochondria; m, microtubules; and sv, synaptic vesicles. Scale bars = 0.1 μm.

3.4. Dominant-negative interference of AP-2 function in human cells selectively impairs transferrin receptor endocytosis

The role of AP-2 in human cells in culture was analyzed using a dominant-negative approach. The μ2 subunit of AP-2 had been previously shown to interact with tyrosine-based sorting motifs of the YXXØ-type (Ohno et al., 1995). Mutation of D^{176} to A (Aguilar et al., 1997) or W^{421} to A (Nesterov et al., 1999) in μ2 were found to block this interaction. When μ2-D^{176}A-W^{421}A was expressed in HeLa cells under the control of a tetracycline-inducible promoter, the mutant subunit was found to replace the endogenous μ2 subunit in the AP-2 complex. Endocytosis of the transferrin receptor was severely impaired in these cells (Nesterov et al., 1999), thus demonstrating that AP-2 mediates rapid receptor endocytosis in human cells.

3.5. Summary: role of AP-2 in endocytosis

The genetic analyses of AP-2 function in various organisms again illustrate the dichotomy between the requirements in yeast and higher eukaryotes. In *S. cerevisiae*, AP-2 is not essential for endocytosis or cell viability, nor does it seem to interact physically or genetically with clathrin. In higher eukaryotes, on the other hand, AP-2 plays a critical role in clathrin-mediated endocytosis in different cell types and is required for the development of multicellular organisms. These findings from genetic analyses are in agreement with the role of AP-2 in endocytosis previously suggested by extensive biochemical and morphological studies of mammalian cells.

4. AP-3

4.1. Missorting of alkaline phosphatase in S. cerevisiae strains deficient in AP-3

Unlike the disruption of AP-1 and AP-2 subunit genes, disruption of the genes encoding each of the four subunits of AP-3 [Apl5p (δ), Apl6p (β3), Apm3p (μ3), Aps3p (σ3)] in *S. cerevisiae* did yield an observable phenotype. The AP-3 mutant strains were viable but exhibited a selective mislocalization of the vacuolar transmembrane protein, alkaline phosphatase (ALP) and the vacuolar t-SNARE Vam3p to cytoplasmic vesicles and tubules (Cowles et al., 1997a; Piper et al., 1997; Stepp et al., 1997). In contrast, transport of CPY and carboxypeptidase S (CPS) to the vacuole were not affected in the AP-3-deficient cells. These observations can be interpreted in the context of the existence of two major pathways for the biosynthetic delivery of hydrolases to the vacuole in *S. cerevisiae*. The first pathway, followed by CPY and CPS, is referred to as the 'CPY pathway' and involves the coat proteins clathrin, Gga1p, Gga2p, and AP-1, as discussed above (see Section 2.1) as well as a large number of gene products involved in vesicle formation or fusion such as Vps45p and Pep12p (Vida et al., 1993; Cowles et al., 1997b). The second pathway, followed by ALP and Vam3p, is known as the 'ALP pathway' and is independent of clathrin, Gga1p, Gga2p, AP-1, Vps45p and Pep12p. The phenotype of the AP-3 deficient strains is consistent with a specific involvement of AP-3 in the ALP pathway. TGN-derived vesicles containing associated AP-3 have been isolated and found to be 50–130-nm in diameter. These vesicles have also been found to contain the protein Vps41p, which may fulfill a role akin to that of clathrin (Rehling et al., 1999). AP-3-coated vesicles are thought to dock to and fuse with the vacuole directly, without passage through a pre-vacuolar compartment.

4.2. D. melanogaster AP-3 mutants exhibit pigmentation defects

The involvement of AP-3 in transport of specific cargo molecules to the *S. cerevisiae* vacuole is in line with observations made in other model organisms. Studies in *D. melanogaster* were in fact the first to implicate AP-3 in the biogenesis of lysosome-related organelles. Mutations in the genes encoding the δ (Ooi et al., 1997; Simpson et al., 1997), β3 (Kretzschmar et al., 2000; Mullins et al., 2000), μ3 (Mullins et al., 1999), and σ3 (Mullins et al., 1999) subunits of AP-3 were identified in the *garnet*, *ruby*, *carmine*, and *orange* pigmentation mutants, respectively. The AP-3 mutant flies displayed abnormal eye color and severe reductions in the number of granules containing pteridine and ommochrome pigments in the eye pigment cells (Figs. 4A–E). Since pigment granules are biogenetically related to lysosomes, these observations hinted at a role for AP-3 in the sorting of specific cargo proteins to lysosome-related organelles.

AP-3 mutant flies were found to have normal numbers of synaptic vesicles in retinula cells as well as normal performance in tests for locomotor activity and olfactory responses (Mullins et al., 2000). However, more specific behavioral assays revealed subtle defects in neurological activity. Kretzschmar et al. (2000) found a defective behavior of adult *ruby* and *garnet* flies in a visual test for alternating pattern fixation. Moreover, these flies had an increased walking speed. This suggests that AP-3 could be involved either in the biogenesis of a minor subpopulation of synaptic vesicles or in the development of some component of the nervous system.

4.3. Pigmentation, bleeding and neurological defects in mice deficient in AP-3

The connection of AP-3 to the biogenesis of lysosome-related organelles was further emphasized by studies on mammals. Two mouse coat color mutants, mocha and pearl, were found to carry mutations in the genes encoding the δ and β3A subunits of AP-3, respectively (Kantheti et al., 1998; Feng et al., 1999). The mice exhibited hypopigmentation of the coat and eyes (Figs. 4F,G), prolonged

bleeding, and lysosomal abnormalities. These phenotypic characteristics arose from defects in the biogenesis of melanosomes, platelet dense bodies and lysosomes, respectively. Mocha mice also displayed inner ear degeneration leading to balance problems and deafness, as well as neurological defects, such as abnormal theta rhythms and seizures (Kantheti et al., 1998). Miller et al. (1999) characterized the neurological phenotype of mocha mice as hyperactivity, an epileptiform electroencephalogram, and changes in the basic function of the hippocampus. Furthermore, missorting of the zinc transporter ZnT-3 in mocha mice led to a lack of vesicular zinc in axon terminals of the neocortex and the

hippocampus (Kantheti et al., 1998). This absence of zinc in the mossy fibers of mocha mice, however, did not cause changes in paired-pulse facilitation, frequency facilitation or long-term potentiation when compared to wild type mice (Vogt et al., 2000). Thus, it is currently unclear to what extent the neurological defects are due to the depletion of vesicular zinc in certain parts of the brain.

Pearl mice displayed reduced visual sensitivity in the dark-adapted state as a consequence of the lower density of melanosomes in the retina (Balkema et al., 1983). Unlike mocha mice, however, pearl mice had normal hearing and balance, and no obvious neurological abnormalities. This
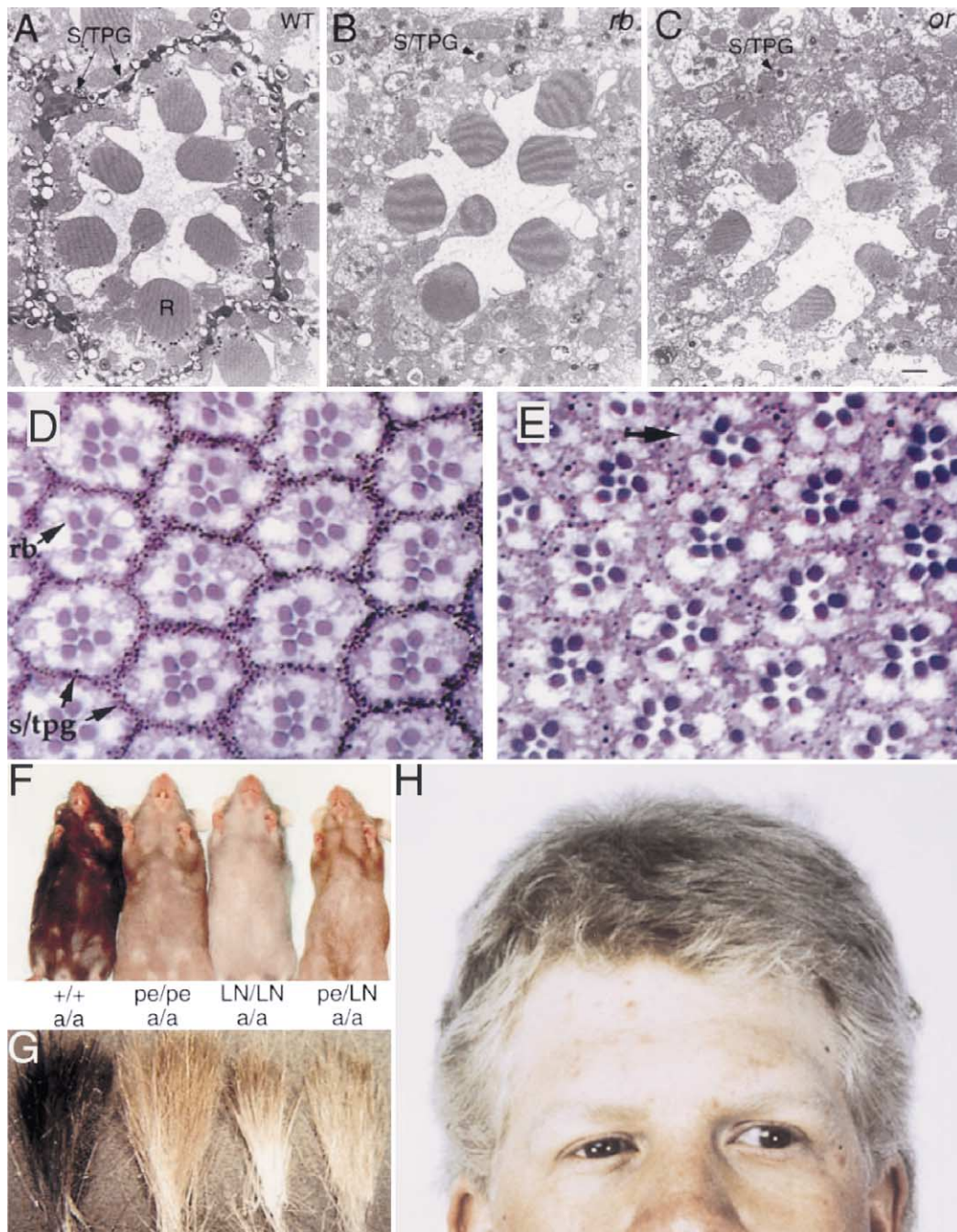
difference could be explained by the expression of the brain-specific β3B subunit isoform in pearl mice, which allows for the assembly of an AP-3B complex containing β3B (and μ3B) only in the brain. The importance of AP-3 complex variants containing brain-specific subunit isoforms has recently been demonstrated by the observation that the formation of synaptic-like microvesicles in an in vitro system was reduced by 50% when cytosol from mice deficient in the μ3B subunit isoform was used (Blumstein et al., 2001). A role for AP-3 in the budding of synaptic vesicles from PC12 donor membranes had previously been suggested from studies in which both forms of AP-3 were removed by pre-absorption with an anti-σ3 antibody. Depletion of AP-3 from brain cytosol resulted in decreased budding of synaptic vesicles (Faundez et al., 1998).

The organellar defects observed in AP-3-deficient mice presumably arise from impaired sorting of some transmembrane proteins to lysosome-related organelles and a subpopulation of synaptic vesicles. Cells derived from mocha or pearl mice, as well as from mice with targeted disruption of the gene encoding β3A (Yang et al., 2000), exhibited increased trafficking of the lysosomal membrane proteins, lamp-1 and lamp-2, to lysosomes by way of the plasma membrane (Dell'Angelica et al., 2000a; Yang et al., 2000).

4.4. Mutations in β3A are the cause of Hermansky-Pudlak syndrome (HPS) type 2 in humans

The phenotypes of pearl and mocha mice are reminiscent of the human genetic disorder, Hermansky-Pudlak syndrome. Like the AP-3 mutant mice, HPS patients exhibit hypopigmentation of the eyes and skin, prolonged bleeding and lysosome abnormalities (Hermansky and Pudlak, 1959). Indeed, two human patients suffering from a particular form of this disease, termed HPS type 2 (Shotelersuk et al., 2000), have been shown to carry mutations in the gene encoding the β3A subunit of AP-3 (Fig. 4H; Dell'Angelica et al., 1999b). The mutant genes were transcribed and the corresponding mRNAs translated, but the resulting protein

products underwent rapid proteasomal degradation. The remaining AP-3 subunits were also degraded, resulting in very low levels of assembled AP-3 in the patient cells.

As in the pearl and mocha cells, lysosomal membrane proteins such as CD63 and lamp-1 were misrouted via the plasma membrane in HPS-2 cells (Dell'Angelica et al., 1999b). Misrouting of the lysosomal transmembrane proteins limp-II and lamp-1 could also be observed upon inhibition of the expression of μ3A in NRK (rat) and HeLa (human) cells, respectively, using an antisense RNA approach (Le Borgne et al., 1998). Recently, the same approach was used to interfere with the trafficking of the quail neuroretina clone 71 gene (Qnr-71) expressed by transfection in HeLa cells. Qnr-71 is sorted to a premelanosomal compartment in retinal pigmented epithelial cells and to an endosomal structure containing the marker EEA1 in HeLa cells. When μ3A synthesis was blocked in HeLa cells, increased re-routing of Qnr-71 via the cell surface was observed (Le Borgne et al., 2001).

The melanosomal defects observed in AP-3-deficient cells could be similarly due to abnormal sorting of melanosomal proteins. In HPS-2 cells, tyrosinase was missorted to multivesicular structures, while TRP-1 was sorted normally to melanosomes (Huizing et al., 2001). MHC class II-invariant chain complexes, however, were sorted normally in human B-lymphoblasts derived from HPS-2 patients (Caplan et al., 2000), as well as in macrophages and B cells from mocha mice (Sevilla et al., 2001). Thus, AP-3 appears to be involved in the sorting of a subset of transmembrane proteins targeted to lysosomes and lysosome-related organelles.

4.5. Summary: role of AP-3 in transport to lysosome-related organelles and neurotransmitter vesicles

The results of the genetic analyses of AP-3 function in various organisms are all consistent with a role in transport to lysosome-related organelles, including the yeast vacuole, fly pigment granules and mammalian melanosomes, platelet dense bodies and some lysosomes. In yeast, the role of AP-3

Fig. 4. Pigmentation defects in AP-3 deficient organisms. Panels A–C [reprinted from Molecular and General Genetics, Vol. 263, Mullins, C. et al., Distinct requirements for the AP-3 adaptor complex in pigment granule and synaptic vesicle biogenesis in *Drosophila melanogaster*, Pages 1003–1014, Copyright© (2000), with permission from Springer-Verlag]: Analysis of visual pigment granules in wild-type and the AP-3 mutant fly strains *ruby* (β3 deficient) and *orange* (σ3 deficient). Electron micrographs depicting an *en face* view of (A) wild type, (B) *ruby* (*rb[1]* allele), and (C) *orange* (*or[1]* allele) mutant *D. melanogaster* eyes at the R7 level show a decrease in the number of electron-dense secondary and tertiary pigment granules (S/TPG) surrounding the photoreceptor cells. R indicates the photosensitive rhabdomeres within a phoreceptor cell. Bar = 1.0 μm. Panels D, E [reprinted from Molecular and General Genetics, Vol. 262, Mullins, C. et al., Defective expression of the μ3 subunit of the AP-3 adaptor complex in the *Drosophila* pigmentation mutant *carmine*, Pages 401–412, Copyright© (1999), with permission from Springer-Verlag]: Analysis of pigment granules in eyes of wild-type and μ3 deficient *carmine* mutant flies. Light photomicrographs of 0.5-μm sections depicting a an *en face* view of (D) wild type, and (E) *carmine* (*cm[1]* allele) mutant *D. melanogaster* eyes reveal a reduction in the number of secondary and tertiary pigment granules (s/tpg) surrounding each group of photoreceptor cells (ommatidium). Panels F, G [reprinted from Journal of Cell Science, Vol. 113, Yang, W. et al., Defective organellar membrane protein trafficking in *Ap3b1*-deficient cells, Pages 4077–4086, Copyright© (2000), with permission from the Company of Biologists Ltd]: Coat color phenotypes of β3A deficient mice strains. (F) Ventral views of C57BL/6 (+/+), C57BL/6-pearl (pe/pe), homozygous β3A knock-out (LN/LN), and heterozygous β3A knock-out/pearl (LN/pe) mice showing that the hair of LN/LN mice is lighter than that of pe/pe mice. Pearl is a naturally-occurring mutation in β3A. (G) Hair tufts from the corresponding mice show less pigment in hair from LN/LN mouse than from pe/pe or LN/pe mice. Panel H [reprinted from The American Journal of Medicine, Vol. 108, Shotelersuk, V. et al., A New Variant of Hermansky-Pudlak Syndrome due to Mutations in a Gene Responsible for Vesicle Formation, Pages 423–427, Copyright© (2000), with permission from Excerptia Medica Inc.]: Hair and skin color phenotype of a β3A deficient HPS patient. The hair of this patient is tan-blond and the skin is lighter than normal.

is limited to sorting of some cargo molecules such as ALP and Vam3p. In mammals, AP-3 appears to participate in the sorting of a larger set of proteins, including lysosomal membrane proteins and some melanosomal proteins. The impact of AP-3 deficiency on the sorting of these proteins is, however, only partial, suggesting the existence of alternative pathways for sorting of these proteins to lysosome-related organelles. These could involve other adaptor protein complexes such as AP-1 and AP-4. The possibility remains, however, that there could be a stricter requirement for AP-3 in the sorting of a smaller set of proteins. The tissue-specific manifestations of AP-3 deficiency in higher eukaryotes could thus reflect the more severe missorting of specialized proteins. In the case of neurotransmitter vesicles, AP-3 could be involved in the sorting of the ZnT-3 transporter. The AP-3B isoform has been implicated in the generation of a subset of synaptic-like microvesicles and the budding of synaptic vesicles from PC12 donor membranes.

An important issue that remains to be elucidated is the intracellular localization of AP-3. The studies done so far indicate that it is localized to the TGN, endosomes, or both, but it is unclear at which of these compartments lysosomal, melanosomal and neurotransmitter/synaptic vesicles proteins are sorted. The requirement for clathrin for AP-3 function in higher eukaryotes is also unclear. Finally, the subset of synaptic vesicles that are generated in an AP-3-dependent fashion and the specific proteins that require AP-3 for sorting into synaptic vesicles remain to be identified.

5. AP-4

To date, no information is available on the function of the AP-4 complex. Its absence in organisms such as *S. cerevisiae*, *C. elegans* and *D. melanogaster* (Boehm and Bonifacino, 2001) suggests that it may not be strictly required for the viability of a unicellular organism or the development of some multicellular organisms. Indirect evidence suggests that the mammalian AP-4 complex might be involved in sorting to the endosomal/lysosomal system in human cells (Aguilar et al., 2001). However, more direct approaches are needed to obtain definitive evidence for its physiological role. Because AP-4 is expressed in *Dictyostelium discoideum*, chickens and mice, targeted interference with the expression of the complex in these organisms should shed light on its role.

6. Concluding remarks

The primary structures of the subunits of AP complexes, as well as their overall architecture, are highly conserved throughout the eukaryotic phylogeny, allowing for the unambiguous assignment of the complexes in organisms as distant as yeasts and mammals. It remains to be fully demonstrated, however, whether these structural similarities reflect a conservation of functions. At present there are both func-

tional similarities and differences that preclude us from reaching definitive conclusions about the generality of AP complex functions. Genetic manipulation of AP-3 has produced the most consistent results among various organisms. All the studies point to a role for AP-3 in protein sorting to lysosome-related organelles and a subpopulation of neurotransmitter/synaptic vesicles, which is not essential for viability. Nevertheless, questions remain about the association of AP-3 with clathrin, its intracellular localization and the identity of the cargo molecules sorted by AP-3 in higher eukaryotes. Genetic analyses of AP-1 and AP-2, on the other hand, have uncovered a dichotomy between yeast and higher eukaryotes. While these two complexes are critical for membrane trafficking in higher eukaryotes, in *S. cerevisiae*, AP-1 and AP-2 are not essential for viability, and the absence of these complexes has little or no effect on protein sorting or other cellular functions. In higher eukaryotes, AP-1 is not essential for viability of isolated cells but is required for development of multicellular organisms. It has not yet been established whether AP-2 is required for cell viability, although it is clearly essential for embryonal development. It is possible that the apparent differences between yeast and metazoans could be reduced to a requirement in single cells vs. multicellular organisms. Perhaps yeasts growing as pseudohyphae or in the wild would manifest a more stringent requirement for AP-1 and AP-2. The exact role of AP-1 remains puzzling. Evidence exists for a role of AP-1 in sorting from the TGN to endosomes (AP-1A, reviewed by Le Borgne and Hoflack, 1998b), endosomes to the TGN (AP-1A, Meyer et al., 2000; Crump et al., 2001; Fölsch et al., 2001), and TGN or endosomes to different domains of the plasma membrane (AP-1B, Fölsch et al., 1999). It is currently unclear whether all of these roles are compatible, although they could be explained by the existence of different AP-1 isoforms. The role of AP-2 in mediating clathrin-dependent internalization from the plasma membrane was well established even before the advent of genetic manipulation procedures. This presumption has been borne out in the genetic analyses of AP-2-deficient organisms and cells. Finally, it will be interesting to ascertain whether AP-4 is important for viability and/or sorting, as its conservation in some organisms would indicate.

Acknowledgements

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